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Short running title: Biochar increases plant productivity by N fertilization

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Initial biochar effects on plant productivity derive from N fertilization

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Abstract

Background and Aim Biochar application to soil is widely claimed to increase plant productivity. However, the underlying mechanisms are still not conclusively described. Here, we aim to elucidate these mechanisms using stable isotope probing.

Methods We conducted two experiments with uniquely double-labelled (^{15}N and ^{13}C) biochar and its feedstock (residue), applied separately at 15 Mg ha^{-1}. Both experiments contained three treatments: biochar amendment (Biochar), unpyrolysed residue amendment (Residue) and a no addition control (Control). Experiment I was a 119 day pot experiment seeded with *Lolium perenne*. Experiment II was a 71 day incubation experiment without plants in which CO_2 and N_2O fluxes were measured.

Results Both Biochar and Residue significantly increased aboveground productivity compared to Control (140 % and 160 %, respectively). Initial N immobilisation was stimulated in Residue, whereas not in Biochar. ^{13}C-CO_2 analysis confirmed that biochar was significantly more recalcitrant than residue. ^{15}N analysis showed that 2 % and 0.3 % of grass N was derived from the amended material in Residue and Biochar, respectively.

Conclusions Our results suggest that biochar-induced yield increases derive from a combination of reduced N immobilization and a moderate N fertilization effect. Although in the short term biochar might offer benefits compared to residue incorporation, it is unlikely that biochar yield gains will be sustainable for the decades to centuries that biochar C can be expected to reside in soil.

Introduction

Interest in biochar has grown considerably since the term was first coined in 2000 (Karaosmanoğlu et al. 2000) and subsequently recognized as a soil conditioner (Lehmann et al. 2006). Biochar is produced through the heating of biomass (feedstock) to temperatures generally exceeding 350 °C, in low to zero oxygen environments (Shackley et al. 2013). It has been repeatedly demonstrated that biochar application to soil can bring benefits in terms of crop yield increases (Jeffery et al. 2011; Liu et al. 2013; Jeffery et al. 2015a). Other studies have shown that it can reduce greenhouse gas (GHG) emissions (Cayuela et al. 2014; Maestrini et al. 2014; Sagrilo et al. 2015), and increase carbon (C) storage in soils (Gurwick et al. 2013), thereby potentially mitigating climate change (Woolf et al. 2010). However, negative effects have also been reported (Mukherjee and Lal 2014), including negative effects on crop yields (Singla et al. 2014; Nelissen et al. 2015).

Despite the growing body of research, the mechanisms behind observed effects following biochar application to soil remain poorly understood. This is largely due to a lack of appropriate experimental controls, as well as the systems-level research approach generally adopted (Jeffery et al. 2015b). A mechanistic understanding of biochar impacts is vital to allow effective predictions regarding biochar soil amendment and its consequences for soil-based ecosystem services including crop productivity. This will aid maximisation of the potential benefits of biochar application to soil while concurrently minimising trade-offs (Crombie et al. 2015; Jeffery et al. 2015b)

One potential mechanism underlying crop yield increases following biochar application is a fertilization effect. This has been shown for potassium (K) which is present in the ash component of biochar (Mia et al. 2014; Oram et al. 2014). Besides providing nutrients, biochar may also affect nutrient cycling and leaching of nutrients in indirect ways (Spokas et al. 2012; Clough et al. 2013). Two extensive reviews on the effects of biochar application to soil on N dynamics (Clough et al. 2013; Cayuela et al. 2014) suggest that one of the main mechanisms is adsorption leading to reduced N

leaching. This is particularly true for high temperature (>600 °C) biochars and for NO_3. Conversely, NH_4^+ retention appears more dependent on the type of feedstock than on pyrolysis temperature (Karaosmanoğlu et al. 2000). Biochar has also been shown to interact with denitrification through its function as an electron shuttle during redox reactions (Cayuela et al. 2013).

Few studies have aimed to investigate the bioavailability of N from biochars beyond quantifying hydrolysable organic N (Clough et al. 2013). Meta-analyses have not found significant differences in yield effects with biochar applied alone or in combinations with fertilizers, either organic or inorganic (Jeffery et al. 2011; Liu et al. 2013). On an individual study level there is some evidence that biochar application to soil can decrease the apparent nitrogen recovery (ANR) of plants (Nelissen et al. 2015). The suggested mechanism for this effect was N immobilisation. However, this effect is dependent on pyrolysis conditions and type of feedstock, as they lead to different propensities for labile compounds to remain on the surface of biochar particles post production (Cornelissen et al. 2005). The application of stable isotope ^{15}N probing provides a means of quantifying the relative and absolute uptake of N from materials amended to soil (Bedard-Haughn et al. 2003), such as biochar, and so quantifying bioavailability of this key plant nutrient.

Besides interactions with mineral N, biochar application to soil has also been shown to interact with soil organic matter (SOM). These interactions include accelerated turnover of SOM (i.e. positive priming; Wardle et al. 1999), reduced turnover of SOM (i.e. negative priming; Zimmerman et al. 2010) and no effect (Sagrilo et al. 2015). The application of ^{13}C labelled biochar can provide insights into the contribution of C pools to CO_2 fluxes as well as into immobilization / decomposition effects related to N availability (Boschker et al. 1998). Further, through combination with ^{13}C phospholipid fatty acid (PLFA) analysis, the main microbial groups able to utilise substrates can be identified, potentially providing insights into microbial-based mechanisms (Boschker et al. 1998).

Here, we utilise a double-labelled (^{13}C and ^{15}N) biochar and its feedstock to investigate the effects of biochar application to soil on N availability and relate C and GHG dynamics. Work conducted here was focussed on grasslands, which have been largely overlooked in biochar research despite suggestions that application to grassland will be required to maximise the GHG offsetting capabilities of biochar (Woolf et al. 2010). Through the use of unpyrolysed feedstock as a positive control we aim to elucidate biochar effects *per se*, i.e. those that are beyond what would have been observed with the application of the feedstock alone. To do so we will test the hypothesis that plant productivity increases following biochar application to soil derive from a fertility effect. If accepted, this suggests that yield effects may not last for as long as the residence time of C in soil, often estimated to be in the range of decades to centuries (Lehmann et al. 2006). Rather, they will last until available nutrients are utilised and become limiting locally once more.

Materials and Methods

This project was focussed on grasslands and so grassland species were used both for the feedstock and for the plants grown. *Plantago lanceolate* is a common plant in grasslands and is fast growing with broad leaves meaning it produces biomass relatively quickly. It was also applied in unpyrolysed form as a positive control (hereafter Residue). The feedstock was isotopically enriched with ^{13}C and ^{15}N as described below. These materials were used in two experiments that used the same homogenised soil: a greenhouse experiment with *Lolium perenne* grown in pots (Experiment I) and an incubation experiment without plants in a climate controlled room to quantify GHG fluxes (Experiment II).

Isotopically labelling biomass

89 Biomass (*Plantago lanceolata*) was grown in a growth chamber in a vermiculite substrate. Key
90 characteristics can be found in Table 1. During the growth period it was pulse-labelled with ^{13}C-CO_2,
91 following the method of Bromand et al. (2001). Biomass was labelled with ^{15}N through fertilization
92 with $Ca(^{15}NO_3)_2$ added to a fertilizer solution applied to the vermiculite daily to achieve an
93 enrichment approx. 58 % atom. Aboveground biomass of *Plantago lanceolata* was harvested twice,
94 at pre-flowering stage. This was done to ensure only leaves were included and no stems or flowers,
95 thereby reducing the heterogeneity of the feedstock. Harvests were performed by cutting plants
96 back to approx. 2 cm above the surface of the vermiculite, after 5 weeks for the first harvest and 7
97 weeks for the second. After harvest, biomass was oven-dried at 60 °C for 24 hours. The dried
98 biomass of both harvests was ground to 2 mm, combined and mixed. A sub-sample of approximately
99 70 % of the biomass was pyrolysed to produce biochar (pyrolysis under N_2, max temp 400 °C,
100 residence time 30 min; Aberystwyth University, Wales). The remaining 30 % of the biomass was used
101 for the Residue treatments described below. Analyses of biochar and residue from which it was
102 produced were performed using a Pyris 1 thermogravimetric analyser (TGA) (Perkin–Elmer,
103 Massachusetts, USA; Hodgson et al. 2011). In short, samples were pyrolysed under nitrogen at a flow
104 rate of 20 mL min^{-1} using the following temperature program: Heated from 40 to 105 °C at 10
105 °C min^{-1}; held at 105 °C for 10 min; heated from 105 to 905 °C at 10, 25, and 100 °C min^{-1}; held at 905
106 °C for 15 min; cooled from 905 to 105 °C at 25 °C min^{-1}. A proximate analysis was performed on the
107 TGA data to calculate the relative proportions volatiles, fixed carbon and ash (wt. %). Volatiles were
108 calculated from mass loss occurring between 105 and 550 °C, fixed carbon from 550 °C to 900 °C and
109 ash as the remaining material after heating. An elemental analysis was used for analysis of $H:C_{org}$
110 which is reported as a molar mass ratio.

111 For the C:N ratio, ^{13}C and ^{15}N content analysis, three replicates (2 mg) of both residue and biochar
112 were placed into individual tin capsules and analysed using a PDZ Europa ANCA-GSL elemental
113 analyser interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd.; Cheshire,

UK) at The Stable Isotope Facility of UC Davis, USA. Key characteristics of both the residue and the biochar can be found in Table 1.

Soil

Topsoil (top 10 cm) was collected from a nature restoration grassland area on the Veluwe, in Gelderland, Netherlands (52.059826N, 5.751354E) on 11th March 2014. The site is located on an ice pushed ridge formed during the Saalien Ice Age. The soil is characterised as a "holtpodzol" on coarse sand (gY30; Stiboka, 1975: map 40 W). The area was used as arable field until 1995 and had last been used to grow maize in 1995. Previous to that cropping had included cycles of sugar beet, potatoes and oats. Collected soil was sieved to pass 4 mm and thoroughly mixed to ensure homogenisation. After homogenisation the soil was split into two parts to be used for Experiments I and II. Soil characteristics were determined in Mia et al. (2014) and Oram et al. (2014). Further information on methods for soil analysis can be found in those studies. Key soil characteristics are presented in Table 2.

Experiment I – Plant growth

Soil for each treatment was amended with biochar and residue each at a rate equivalent to 15 t ha^{-1}, incorporated into the top 10 cm of soil produced with five replicates. Soil was packed into 9.5 cm diameter 0.5 L polypropylene pots (505 g dry weight (dw) of soil, packed to a dry bulk density of 1.2 g cm^{-3}). The control consisted of unamended soil packed to the same bulk density. A 1-cm deep layer consisting of 94 g of soil (i.e. without biochar or feedstock) was added to the surface of pots to function as a germination layer as biochar has previously been shown to occasionally inhibit germination. All treatments were replicated five times, totalling 15 pots, set up in a completely randomised design. The experiment was performed in a greenhouse (average 60 % relative humidity; average temperature 21 °C) of Wageningen University, The Netherlands.

In each pot, 10 wild type *Lolium perenne* (diploid) seeds were sown at a seeding rate equivalent to 27 kg ha^{-1}. Owing to reduced germination in some pots, pots were reseeded after a week in order to achieve 10 plants per pot.

After germination, pots were fertilized at rates equivalent to 30 kg P ha^{-1} and 140 kg K ha^{-1} (KH_2PO_4 and K_2SO_4). Applications of fertilizer were spread over four days to minimise the risk of burning the seedlings. No N fertilizer was added to any of the pots. Water was added following fertilisation to bring all the pots to 60 % water-filled pore space (WFPS) and to ensure that the fertilizer moved deeper into the soil. The pots were then watered daily and maintained gravimetrically at 60 % WFPS.

Aboveground biomass was harvested at Day 35 by cutting the plants back to approximately 2 cm above the soil surface. The second, third and fourth harvest of biomass were respectively on Day 63, 91 and 119. Biomass was oven dried at 60 °C for at least 48 h and weighed. Subsequently, all biomass from each pot was combined, ground and ball milled. A representative subsample (approx. 2 mg) of aboveground biomass was then isotopically analysed for ^{13}C and ^{15}N content as described below. Belowground biomass was collected by washing roots over a 2 mm sieve to remove soil particles. Roots were then oven-dried and weighed as described above.

Experiment II – Soil gas fluxes

Experiment II consisted of the same three treatments as Experiment I but without plants. Pots (polypropylene 0.5 L – 6.6 cm diameter) were packed with 200 g soil dry weight (dw) to a dry bulk density of 1.2 g cm^{-3}. All treatments were replicated 5 times, totalling 15 pots. The pots were placed on a table in a completely randomized design in a climate-controlled room at 20 °C and maintained at 60 % WFPS.

On days 1, 2, 5, 8, 16, 22, 29, 36, 43, 50, 57, 64, and 71, CO_2 and N_2O gas samples were taken and fluxes were measured. This was done one hour after closing the pot with a lid containing two septa.

Two separate gas samples (7 ml each) were taken with a syringe and injected into pre-evacuated 4.5ml borosilicate vials for analysis of ^{13}C-CO_2 and ^{15}N-N_2O content. Soil gas fluxes were then quantified following a standard procedure with photoacoustic gas monitor (Brüel & Kjær, Monitor Type 1302; Nærum, Denmark; Velthof et al. 2002). Daily fluxes (ppm) were converted to mg CO_2-C h^{-1} m^{-2} and µg N_2O-N h^{-1} m^{-2} and to cumulative fluxes in g CO_2-C m^{-2} and mg N_2O-N m^{-2} assuming linearity of flux rate between each measurement day.

Stable Isotope analyses

All isotope analyses were performed at The Stable Isotope Facility of University California, Davis. The ^{13}C content analyses were performed using a ThermoScientific PreCon-GasBench system interfaced to a ThermoScientific Delta V Plus isotope ratio mass spectrometer (ThermoScientific, Bremen, Germany). The ^{15}N content analyses were performed using a ThermoFinnigan GasBench + PreCon trace gas concentration system interfaced to a ThermoScientific Delta V Plus isotope-ratio mass spectrometer (Bremen, Germany). Percentage C and N derived from the biochar and feedstock were calculated using ^{13}C and ^{15}N gas values and applying the equation of Bedard-Haughn et al. (2003). These percentage values were then used to calculate the g CO_2-C m^{-2} and mg N_2O-N m^{-2}, as a proportion of the total flux, derived from the Biochar and Residue.

On Day 71, the soil from each pot was sieved to pass 4 mm, homogenised by thorough mixing and split into sub-samples for analysis. A representative subsample (50 mg) of the soil was analysed (^{13}C and ^{15}N content) as described below. The pH and EC was determined after shaking each sample (5 g) for 1 h with demi-water (1:5 w/v).

Microbial biomass ^{15}N

Determination of microbial biomass N (MBN) was undertaken via an extension of the chloroform fumigation extraction (Vance et al. 1996). In short, soil (20 g) was shaken for 1 hour with 80 ml 0.5 M

KCl following 24 hours of fumigation. After shaking, extracts were filtered to pass 0.45 µm. The difference in total soluble N content (TSN) between the fumigated and non-fumigated soil in the KCl extract was used to calculate the MBN. Microdiffusion was used to quantify the ^{15}N content of MBN (Stark and Hart 1996) through the analysis of ^{15}N that was obtained on the filter. Each glass microfiber filter used for the microdiffusion was put in tin capsules and analysed for ^{15}N content as described above.

^{13}C PLFA

The phospholipid fatty acid (PLFA) extraction and analysis as outlined by Bligh and Dyer (1959) and extended upon by Zelles (1999) was utilised to determine microbial community level phenotypes. Extractions were performed using 5-g aliquots of soil for each sample. Extracted PLFAs were analysed by gas chromatography using an HP 5 column on a G2070AA Chemstation, Model 6890N, gas chromatography appliance (Agilent Technologies, Santa Clara, California, USA). Peaks were compared with known retention times on the basis of a Supelco 26 peak standard to identify individual PLFAs (Sigma-Aldrich Ltd, Poole, Dorset, UK). The fungal:bacterial ratio was calculated using 18:2ω6 (fungal biomarker) divided by the summed % mol of biomarkers i15:0, ai15:0, 15:0, i16:0, 16:1ω7t, i17:0, ai17:0, 17:0, 18:1ω7 and cy19:0 as an expression of total bacterial abundance (Frostegård & Bååth, 1996). $\delta^{13}C$ values were measured on a Finnigan Delta-S gas chromatograph–isotope ratio monitoring mass spectrometer (GC-IRMS) as described in Boschker (2004). The increase in $\delta^{13}C$ values of PLFAs in the treatments compared to the control indicates microbial uptake of labelled C from the amended material.

Data analysis

For statistical analyses, SPSS Statistics 19 (IBM) was used with the exception of Principal Component Analysis used to analyse PLFA profiles, which was performed using R Studio (version 0.99.903) with the Vegan package. The effects of the treatments (Biochar, Reside and Control) on cumulative fluxes

of CO_2 and N_2O, the ^{13}C and ^{15}N content, pH and EC of the incubation soil, N content of the incubation soil and the microbial biomass N were compared using a one-way Analysis of Variance (ANOVA). Individual comparisons were performed using a Tukey's HSD *post hoc* test. The effects of the treatments on plant performance and ^{13}C and ^{15}N uptake by plants were compared using ANCOVA with the number of plants per pot included as a covariate. The treatment effects on daily CO_2 and N_2O fluxes and the ^{13}C and ^{15}N content were tested with a repeated measures ANOVA.

Results

Experiment 1: Plant growth

Despite the germination layer an average of 5.3 (S.E. 1.16), 5.2 (S.E. 1.2) and 8.4 (S.E. 0.68) plants germinated in Biochar, Residue and Control, respectively. Aboveground biomass production of *L. perenne* (dry weight) was significantly reduced by 89 % in the first harvest of Residue compared to Control (Fig. 1). However, biomass production in Residue was significantly higher than Control over the next three harvests. On average, biomass production was approx. 40 % and approx. 60 % higher than Control for Biochar and Residue, ($P = 0.001$ and $P = 0.01$, resp.). Belowground biomass increased significantly in Biochar by 115 % compared to Control; no significant difference was observed in belowground biomass production in Residue compared to Control. (Fig. 1). Differences in the shoot:root ratio between treatments were close to significant ($P = 0.064$).

Significant differences in N uptake were observed between Biochar and Residue ($P < 0.001$). Overall, more N was taken up by plants from the amended material in Reside than in Biochar (Fig. 2 a & b). At first harvest, significantly more N was taken up from the amended material in Biochar than in Residue in absolute terms ($P < 0.001$; Figure 2a). However, as a proportion of total N taken up over all harvests, significantly more plant N was derived from the amended material in Residue than in Biochar ($P<0.001$; Fig. 2b). After the first harvest, approximately 2 to 2.5 % of N taken up by plants in

Residue was derived from the amended material. For Biochar, this was less than 0.5 % for all harvests (Figure 2b). After four harvests, biochar N accounted for approx. 0.4 % of total plant N whereas for residue N this was approx. 2 %.

Experiment 2: Soil gas fluxes

Cumulative N_2O fluxes from Biochar did not vary significantly from Control throughout the timeframe of the experiment (P = 0.9; Fig. 3a). Residue resulted in a significantly greater release of N_2O than either Control or Biochar up to Day 5 (P < 0.001; Figure 3a). The N_2O flux from Reside was reduced greatly after this initial flush but increased again at Day 64 until the end of the experiment.

After 71 days, at the end of the incubation experiment, approximately 0.3 % of amended N had been lost as N_2O from the amendment in Residue, compared to 0.05 % from Biochar (Fig. 3). N-loss from the amended material in Biochar as N_2O was significantly lower than from Residue (P < 0.01) suggesting decreased availability of N from biochar than residue. However, N_2O did not represent a significant source of N loss from either experimental treatment.

% %By Day 2, significantly higher cumulative CO_2 fluxes were measured in Residue compared to Biochar and Control (P = 0.001); this difference increased throughout the incubation period. There was no significant difference in total cumulative fluxes between Biochar and Control (P = 0.96; Fig. 3b). Significantly more C was lost from Residue by Day 5 (Fig 3d; P = 0.008); by the end of the experiment approx. 20 % of the applied C was lost from Residue as CO_2 compared to approx. 2 % loss from Biochar (Fig. 3d). However, the rate of C loss from biochar reduced greatly after the initial flush.

By the end of the experiment, microbial biomass nitrogen (MBN) was four times higher in Residue than in Biochar or Control (P <0.001; Fig. 4a). There was no significant difference in MBN between Biochar and Control (P = 0.78; Fig. 4a). Stable isotope analysis showed that for the Residue treatment

252 approximately 25 % of total MBN was derived from the amended material, while this was only
253 approximately 0.3 % for the Biochar treatment (Fig. 4a).

254 The CO_2-C derived from SOM did not differ significantly from Control in either treatment ($P > 0.05$;
255 Fig. 4b). However, significantly more CO_2-C was derived from the amended material in Residue than
256 Biochar (56 % compared to 39 %; $P<0.001$; Fig. 4b). Further, significantly more C was mineralised
257 from SOM in Residue than Biochar (Fig. 4b; $P = 0.03$) showing that both C pools had increased
258 turnover in Residue.

259 PLFA profile analysis of the community level microbial phenotype showed strong discrimination
260 between Residue compared to Biochar and Control where little discrimination was evident (Fig. 5a).
261 Discrimination between treatments occurred mainly in PC1, which accounted for 86 % of variation.
262 The PLFAs most responsible for the observed discrimination between treatments were C16:0
263 (general biomarker for microbial biomass), C18:2ω6c and C18:1ω9c /2ω6t/3ω (saprotrophic fungal
264 biomarkers – note that with the methodology used it was not possible to discriminate between these
265 PLFAs; Fig 5b). The bacterial: fungal ratios were significantly lower in Control (0.03) and Biochar
266 (0.04) compared to Residue (0.3) ($P < 0.01$).

267 The stable isotope enrichment increased significantly from -30.4‰, -32.1‰ and -29.4‰ in Control to
268 8.3‰, 5.5‰ and -0.5‰ in Biochar and 140‰, 87.8‰ and 154.4‰ in Residue for the PLFAs C16:0,
269 C18:2ω6c and C18:1ω9c/2ω6t/3ω respectively (Figure 5c; $P < 0.01$).

270 Discussion

271 The increased plant productivity following soil biochar amendment that we found in this study is in
272 agreement with previous studies (Jeffery et al.2011; Spokas et al. 2012; Liu et al. 2013). However, the
273 use of stable isotope probing allows us to investigate the mechanisms underlying this effect, rather
274 than reporting results at the systems level. Previous biochar studies using soil from the same site

showed that micronutrients in the soil used were not limiting (Oram et al. 2014; van de Voorde et al. 2014). In the present study, soils were fertilized with K and P to ensure that only N would be limiting (Table 2). Total plant production in Biochar and Residue was significantly higher than Control. Concurrently, stable isotope analysis demonstrated uptake of N from both Residue and Biochar. Therefore, the data support the hypothesis that observed differences in biomass production resulted from an N fertilisation from the amendment in the Biochar and Residue treatments. Plant N uptake from the amended material Residue was 7.6 times higher than in Biochar. Differences in biomass production were less apparent. Once N limitation has been alleviated, diminishing returns are expected from further increased N availability (Tillman et al. 2002). This effect was reflected in the biomass data.

Increased root growth was noted in Biochar compared to Residue and Control (Fig. 1). It has previously been reported that plants grown in biochar-amended soils can have increased "rhizosphere zones" compared to controls (Prendergast-Miller et al. 2014). During harvest, we noted that the rhizosphere contained more biochar particles than the bulk soil suggesting that roots may prefer soil containing biochar particles. This may have consequences beyond investigated effects in this experiment. For example, increased rooting may help alleviate the impact of drought as well as aiding nutrient acquisition beyond those included in the amended material. The trigger that led to increased root growth in the presence of biochar remains unclear and a necessary area for further research.

The same amendment application rate was used for Biochar and Residue. As they each contained very similar levels of N (Table 2), similar rates of N were applied to both treatments. However, the C:N of the applied materials differed significantly. It is not yet clear how the C:N stoichiometery interacts with soil processes as it is likely the quality of the C that is important rather than the quantity. In the Residue treatment more than four times as much N was taken up from the amendment than in the Biochar treatment showing enhanced ANR from the amended material in

300 Residue than Biochar. However, initial N immobilisation reduced ANR significantly at the first harvest
301 in Residue. Such immobilisation was not observed in Biochar where ANR was shown to be highest at
302 first harvest (Fig 2a and b) where it then decreased and remained at a consistent level thereafter.
303 Biochar could have been expected to stimulate N immobilisation more than residue when
304 considering only the C:N ratios alone. That this did not occur provides evidence that the C:N ratio of
305 biochar is likely not an effective predictor as to whether that biochar will immobilise N when applied
306 to soil.

307 There are few data on the availability of N from biochar (Clough et al. 2013; Cayuela et al. 2014).
308 Studies that have investigated N dynamics following biochar application have typically focused on co-
309 application of N fertilizer (Spokas et al. 2012; Zheng et al. 2012; Clough et al. 2013), N retention
310 effects of biochar (Spokas et al. 2012; Zheng et al. 2012), or N_2O flux effects (Zheng et al. 2012;
311 Clough et al. 2013). However, no other study to date has quantitatively analysed the bioavailability of
312 N from biochar itself. Our study shows that pyrolysis reduced availability of N for plant uptake in
313 Biochar by >700 % compared to Residue, but that a significant proportion of N remained bioavailable
314 (or mineralisable to available forms). Reduced N bioavailability led to decreased plant productivity in
315 Biochar compared to Residue, while still being greater than Control. Plant biomass productivity was
316 lowest in Control due to N limitation as it received no amendment (i.e. no input of N).

317 The significant decrease in plant biomass in Residue compared to Biochar and Control at the first
318 harvest suggests N immobilisation. This was likely a consequence of the addition of the relatively
319 large amounts of labile C added to this treatment (i.e. plant residue) as reflected in the CO_2 emission
320 rate (Fig. 3). This is also reflected in the fact that very little N was taken up by plants from the
321 amendment in Residue by the first harvest, and that microbial biomass N was significantly larger in
322 the Residue treatment. Isotopic analysis showed that 0.3 % of MBN was derived from the
323 amendment Biochar, compared to 22.5 % in Residue. This agrees with previous work that found little
324 effect of biochar on MBN in contrast to wheat straw (Zhang et al. 2014). This was likely due to the

application of labile C (i.e. plant material) provided substrate that functioned as an energy source allowing microbes to scavenge for N from SOM through nitrogen-mining (Craine et al. 2007).

There was a significant increase in N_2O production from Residue for the first 5 days of the incubation experiment indicative of increased microbial N cycling in this period. After this time, N_2O fluxes decreased greatly suggesting that readily available N in the soilwas immobilised in the microbial community, or denitrification increased due to depletion of O_2 within soil pores driven by respiration of labile C as. By the end of the incubation there was no significant difference in cumulative N_2O emissions between treatments. This result contrasts with numerous studies which have reported a significant decrease (Cayuela et al. 2014; Case et al. 2015), or increase (Clough et al. 2010; Sánchez-García et al. 2014) in N_2O fluxes following biochar application to soil. However, other studies have also reported no effect on N_2O emissions (Suddick et al. 2013), or different effects from the same biochar applied to different soils (Yoo et al. 2012). These contrasting findings emphasise that generalisation of the effects of biochar should be taken with great care and that the results may depend greatly on characteristics of the biochar and soil used. It should be noted that we used a coarse soil (Table 2) and as such our results may differ from experiments that used a fine soils.

Utilisation of ^{13}C isotopes allowed proportional attribution of CO_2 flux to the different carbon pools in biochar and SOM (Boschker et al. 1998). ^{13}C analysis confirmed that a portion of emitted CO_2 was derived from biochar and that the biochar therefore contained a labile component. However, data presented here demonstrate that the C in the biochar was, on the whole, significantly more recalcitrant than the unpyrolysed feedstock with >3 % of amended C lost from Biochar over the course of the experiment compared to 19 % of amended C lost from Residue. We found no evidence of priming of SOM by addition of Biochar and Residue compared to the control. However, mineralisation rates of SOM differed between the Biochar and Residue treatments. This means that while no priming of SOM occurred compared to the control situation with no addition, differential interactions with SOM in terms of priming effects were observed following the application of Biochar

versus Residue. This was likely due to the increased microbial biomass (as suggested by the increased MBN in Residue; Fig. 4a) which is generally correlated with increased decomposition of soil organic matter (Balota et al. 2003; Lee et al. 2003). The decay constant (k) of SOM are usually imperfect representations of first order kinetics (Paul et al. 1996). As such linear extrapolation cannot be undertaken with confidence but the evidence suggests the residence time of the biochar C would be at least an order of magnitude greater than that of residue C.

Biochar application to soil has been shown to stimulate mycorrhizal fungi and their colonisation of plant roots (Warnock et al. 2007). We quantified microbial community effects through phenotypic fingerprinting using ^{13}C PLFA. While PLFA discriminated between the microbial community in Residue compared to Biochar and Control, no strong discrimination between biochar and control was observed. The PLFA 16:1ω5 considered a biomarker for mycorrhizal fungi (Olsson 1995) did not vary significantly between treatments suggesting that, at least in our study, observed yield effects should not be attributed to increased mycorrhizal fungi as has been posited previously (Warnock et al. 2007). However, the plant used in this experiment, *Lolium perenne,* forms a dense rooting system that may not be conducive to mycorrhizal colonisation. Further, the soil is relatively high in P (Table 2), and was fertilised with soluble P, which tends to reduce mycorrhization. As such, different results may have been observed if different plants or different fertilisation regimes were used. The PLFA C16:0, considered a general microbial biomass marker (Bossio et al. 1998) and C18:1ω9c/2ω6t/3ω and C18:2ω6, all considered saprotrophic fungal biomarkers (Frostegård et al. 1996; von Rein et al. 2016), contributed most to the discrimination observed between treatments. The $\delta^{13}C$ of these PLFAs all increased significantly from Control to Biochar to Residue. This further confirms that some of the C in the biochar was labile and so available for microbial utilisation and incorporation into microbial cell membranes. However, considerably more C was incorporated into microbial cell membranes (and likely microbial cells in general) in Residue, as confirmed by the greatly increased $\delta^{13}C$. These data are again consistent with the fertilisation hypothesis. This evidence suggests that saprotrophic

375 fungal biomass increased in Biochar, and much more so in Residue, where it decomposed the
376 amended material and mineralised organic N into plant available forms.

377 Conclusions

378 Our results demonstrate that the observed increases in plant productivity following biochar addition
379 to soil were due to an N fertilisation effect. Stable isotope analysis using ^{13}C confirmed that the C in
380 biochar is considerably more recalcitrant than the feedstock from which it was produced.

381 Pyrolysis strongly reduced the bioavailability of N from the resulting biochar when compared to the
382 initial feedstock. This means that nutrients in biochar are released slowly when compared to the
383 initial feedstock. Further, due to the increased recalcitrance of the C in biochar, application of
384 biochar to soil did not cause N immobilisation. Therefore, application of biochar rather than crop
385 residues may circumvent the need of co-application of synthetic N fertilisers, which are sometimes
386 applied to compensate for the effects of microbial N immobilisation. This study also highlights the
387 need for rigorous controls in experiments to allow distinguishing fertilisation effects (short-term)
388 from the “true” biochar effects, i.e. those effects associated with biochar C that will occur over the
389 entire residence time of that C in the soil. For sustainable application of biochar it is vital to make
390 informed decisions on where best to apply biochar, compost and/or green manures to maximise the
391 potential benefits and minimise the negative impacts. Our results will help decisions makers such as
392 farmers or policy makers to do so.

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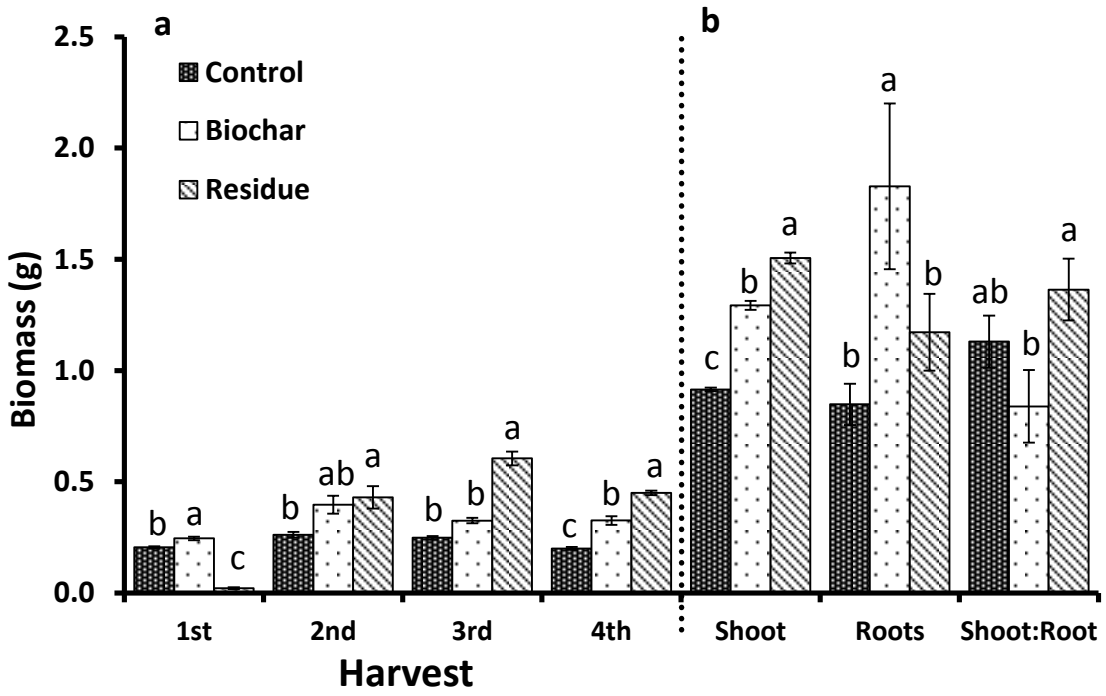
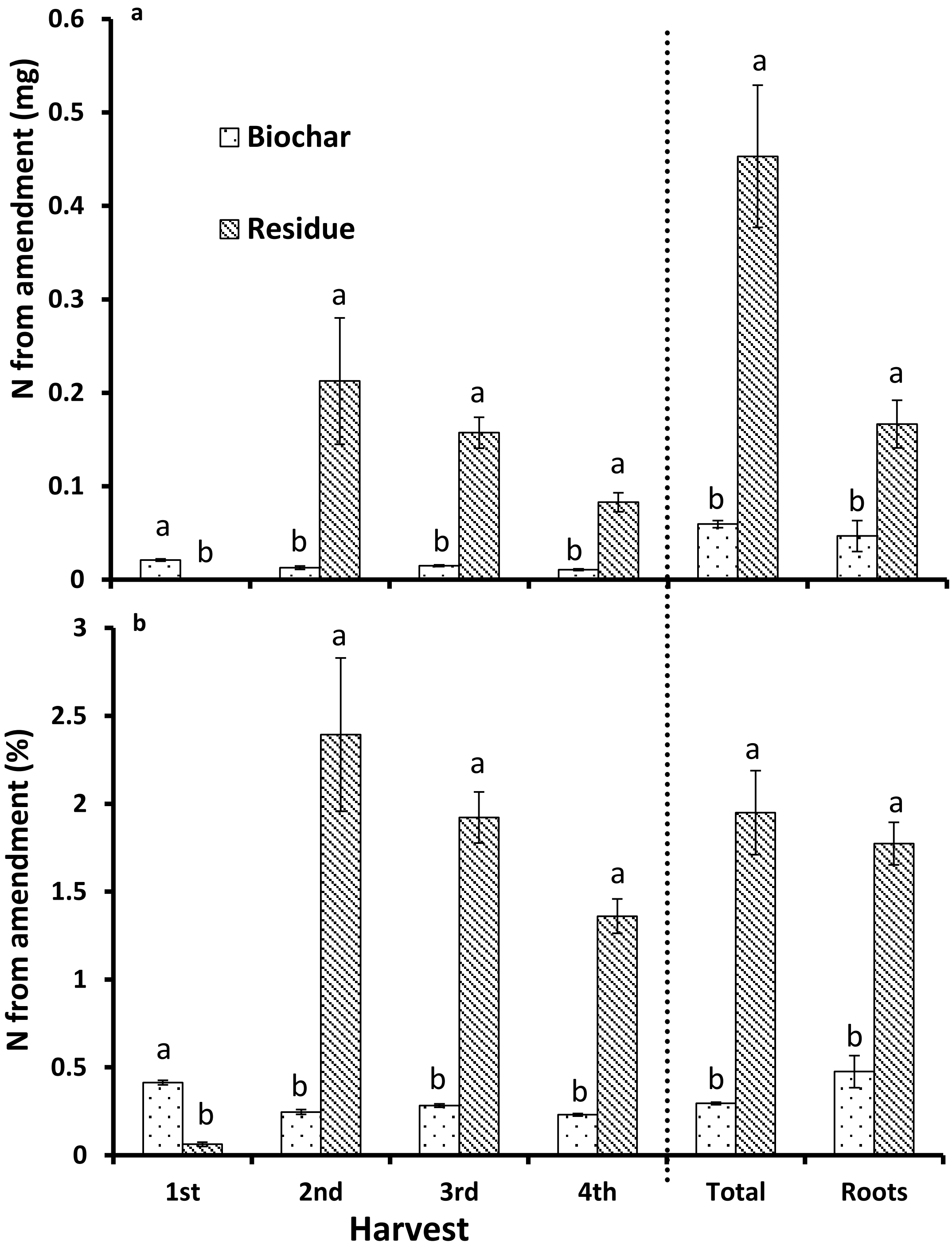
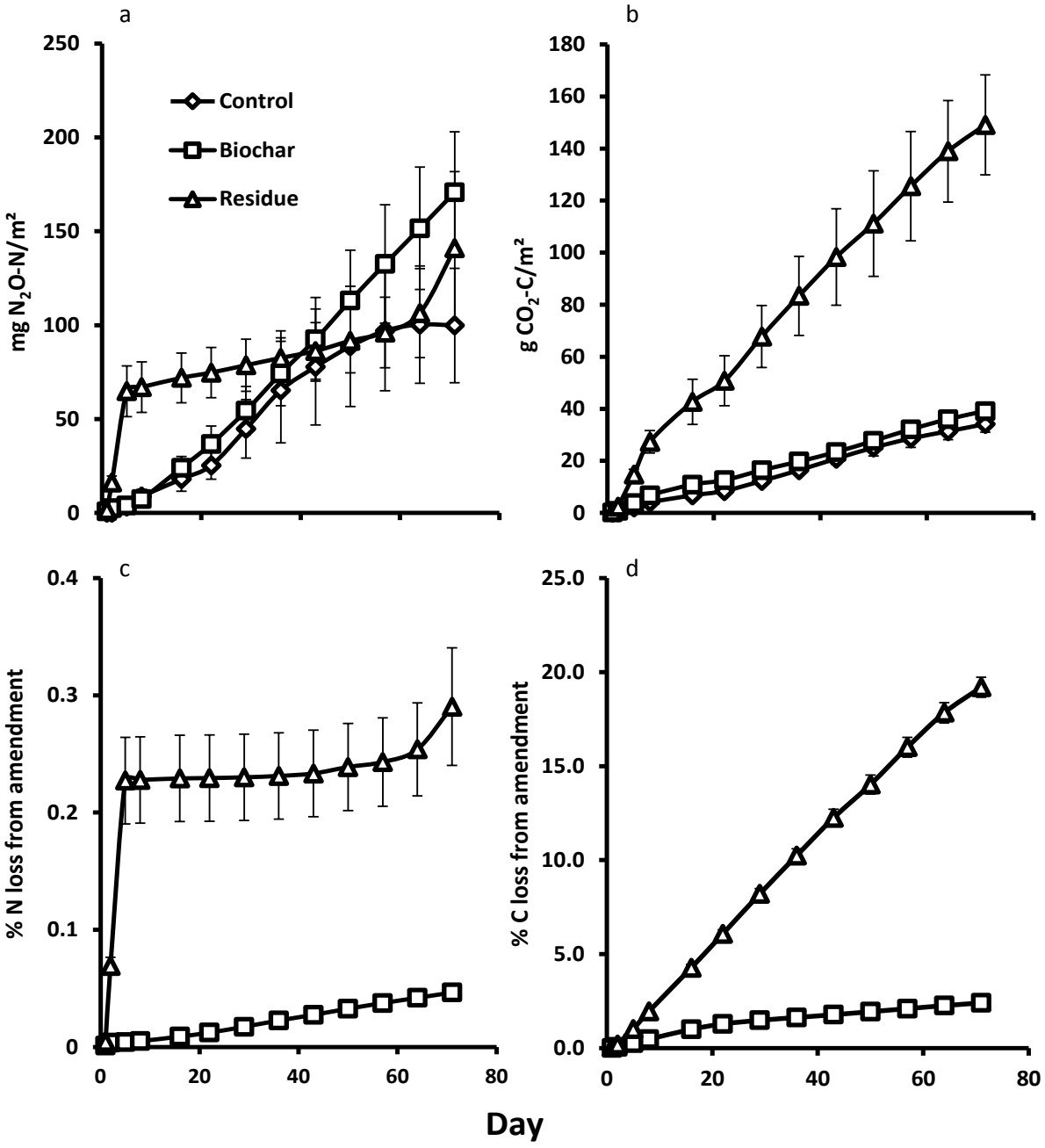
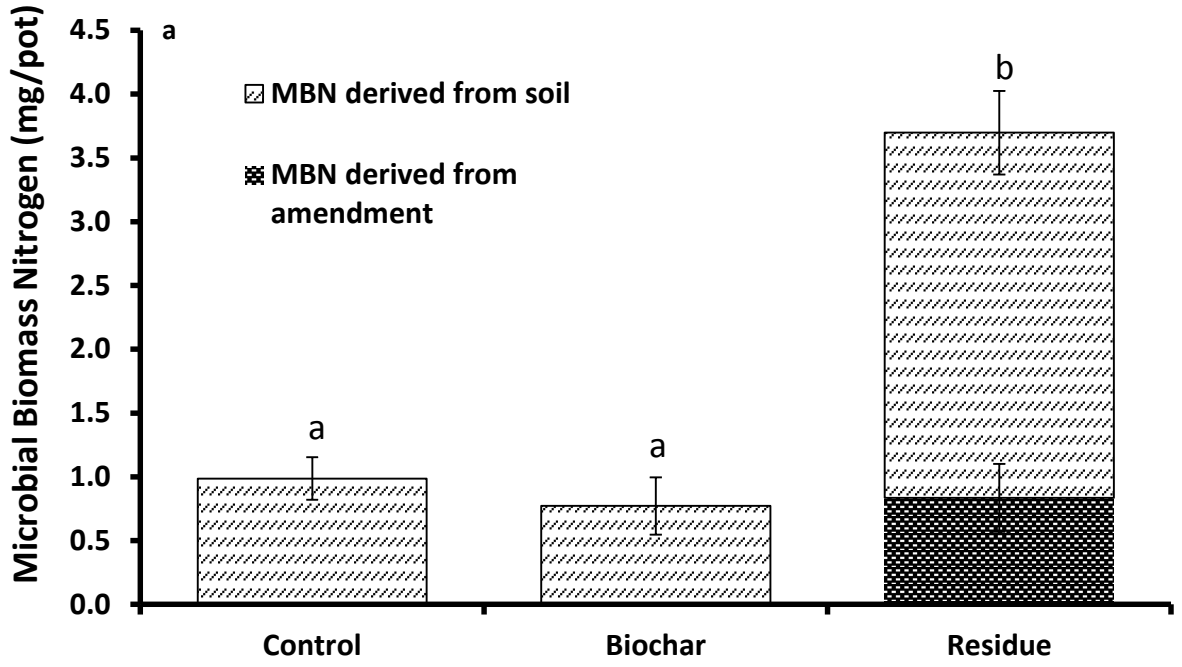
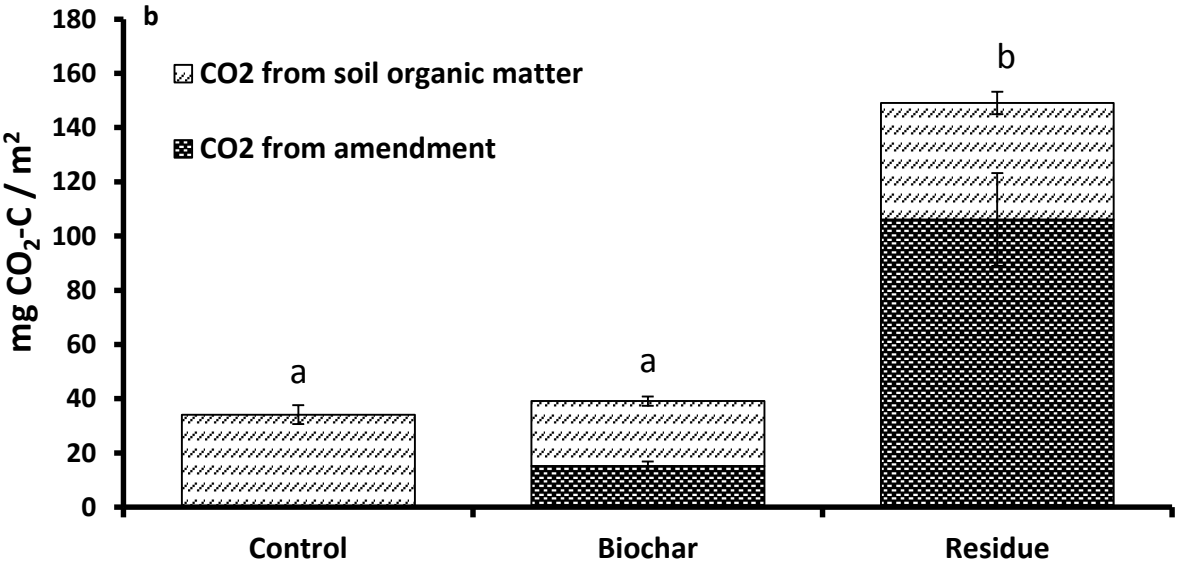
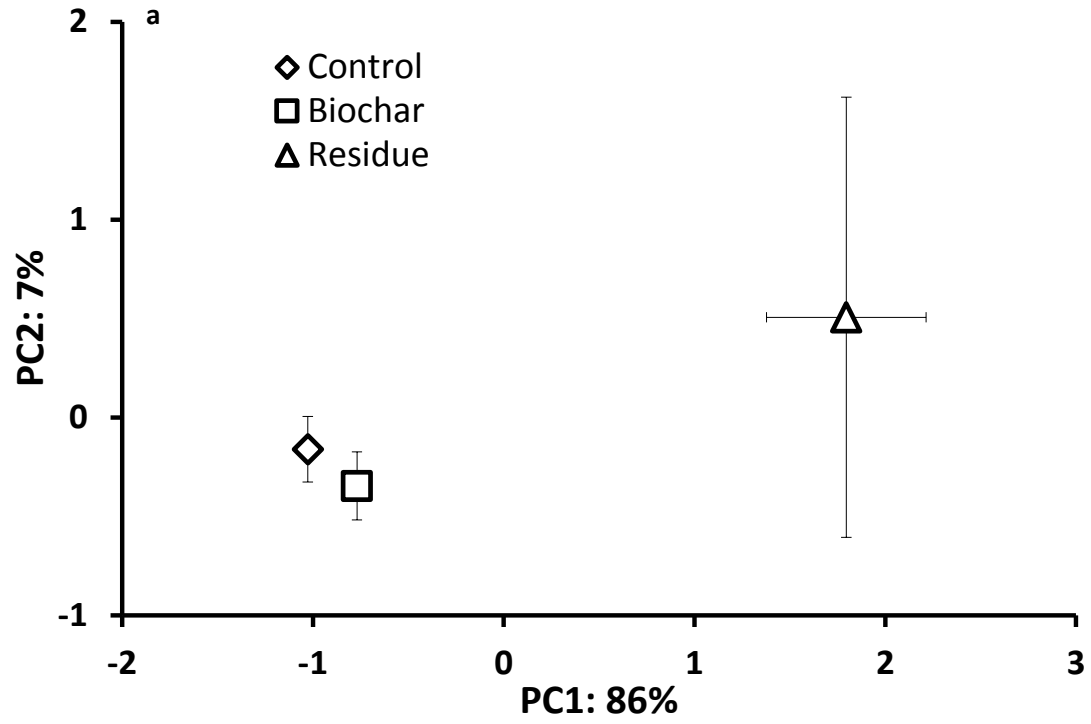

a
b
Control
Biochar
Residue
Biomass (g)
2.5
2.0
1.5
1.0
0.5
0.0
b a c
b ab a
b b a
c b a
c b a
b a b
ab b a
1st
2nd
3rd
4th
Shoot
Roots
Shoot:Root
Harvest

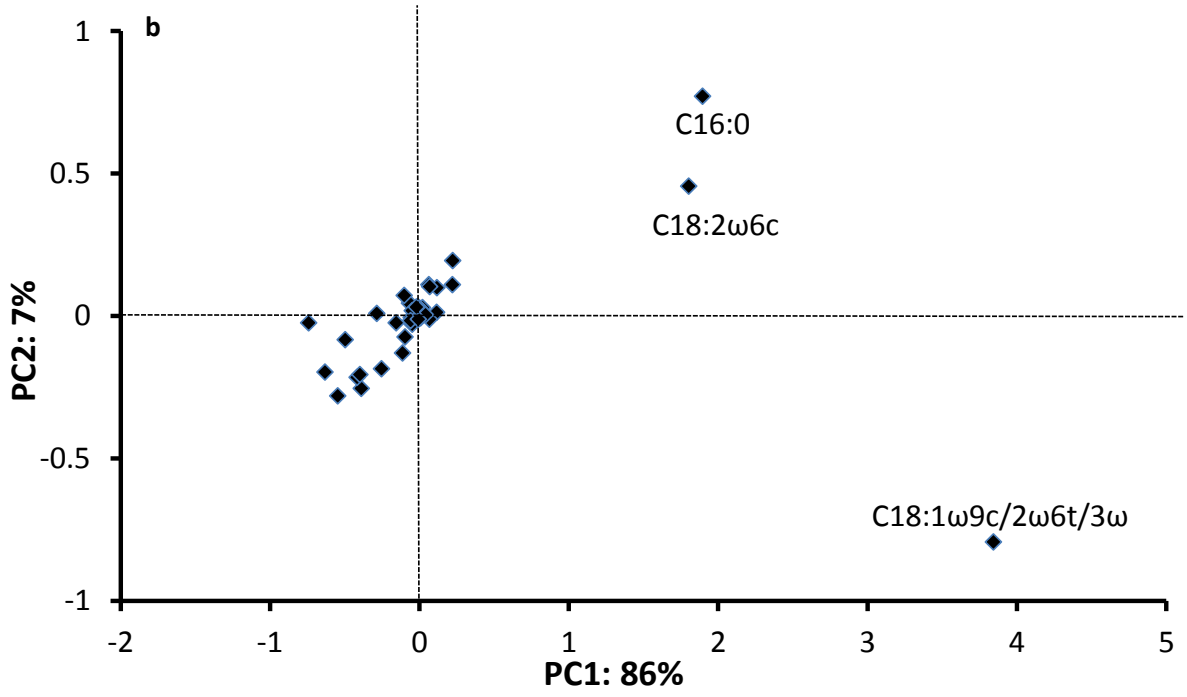

a
N from amendment (mg)
0.6
0.5
0.4
0.3
0.2
0.1
0
Biochar
Residue
b
N from amendment (%)
3
2.5
2
1.5
1
0.5
0
1st
2nd
3rd
4th
Total
Roots
Harvest


a
b
c
d
Control
Biochar
Residue
mg N2O-N/m²
g CO2-C/m²
% N loss from amendment
% C loss from amendment
Day


a
Microbial Biomass Nitrogen (mg/pot)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
MBN derived from soil
MBN derived from amendment
a
a
b
Control
Biochar
Residue


b
mg CO_2-C / m^2
180
160
140
120
100
80
60
40
20
0
CO2 from soil organic matter
CO2 from amendment
a
a
b
Control
Biochar
Residue


a
Control
Biochar
Residue
PC2: 7%
PC1: 86%
2
1
0
-1
-2
-1
0
1
2
3


b
C16:0
C18:2ω6c
C18:1ω9c/2ω6t/3ω
PC2: 7%
PC1: 86%
1
0.5
0
-0.5
-1
-2
-1
0
1
2
3
4
5

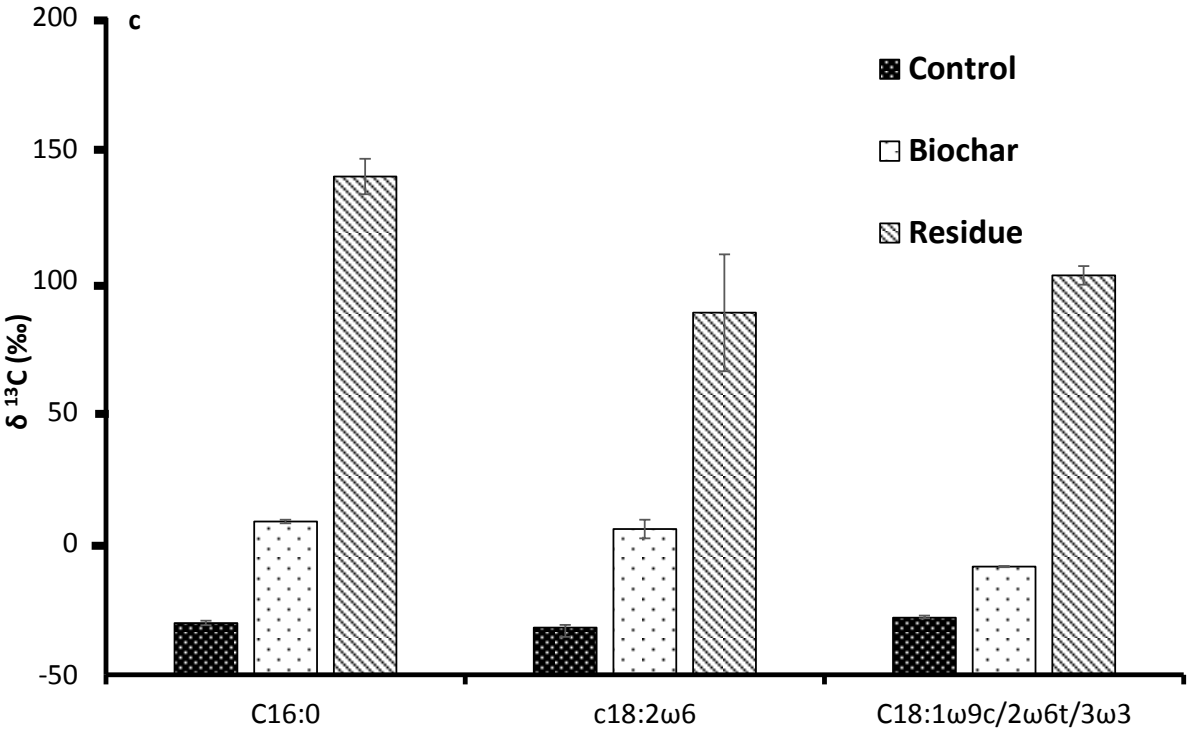

c
200
150
100
50
0
-50
δ 13C (‰)
Control
Biochar
Residue
C16:0
c18:2ω6
C18:1ω9c/2ω6t/3ω3

Figure 1a. *Lolium perenne* biomass (dry weight) produced from each treatment at each of the four harvests after 35, 63, 91 and 119 days; b. total aboveground biomass "Shoot" (i.e. from all harvests combined) and belowground biomass "Roots" (calculated as the average of the belowground biomas produced in each treatment), and the Shoot to Root Ratio after 119 days. Letters show significant differences within each harvest and for plant growth response characteristics. Columns show means, bars show ±standard error (n=5).

Figure 2. (a) Total amount of N taken up from the amended material and (b) proportion of plant N derived from the amended material. Columns show means. Bars show ±standard errors (n=5). N uptake from amendment was significant different between treatments in all cases (P = 0.05). Note that no material was amended to Control and so no data are reported for Control.

Figure 3. Cumulative N_2O (a) and CO_2 (b) fluxes from microcosms in Exp. II over a 71 day incubation period and the percent loss of N (c) and C (d) from the amended material as determined by ^{15}N or ^{13}C analysis. Points show means. Bars show ±standard errors (n=5, apart from Day 2 points where n=4). Note that no material was amended to Control and so no data are reported for Control in (c) or (d).

Figure 4. (a) Microbial biomass N (MBN) as determined by chloroform fumigation extraction with the contribution of each pool of N to microbial biomass N determined by stable isotope ^{15}N 71 days after application of amended material; (b) Cumulative CO_2 emitted from each treatment, derived from each soil C pool using ^{13}C isotope analysis over a 71 day incubation. Columns show means. Shaded columns show the mean contribution of each pool to the total. Bars show ±standard errors (n=5).

Figure 5 (a) A principal component ordination plot of the first two principal components of PLFAs extracted from each sample. Points show mean coordinates of treatment replicates, bars show standard errors (n=5); (b) loading plot in which the PLFAs which contribute most to the discrimination between treatments are labelled; (c) $\delta^{13}C$ profiles of the three PLFAs the contributed

most to the discrimination observed between treatments. Larger bars represent more uptake of applied enriched ^{13}C material. Columns show means. Bars show ±standard errors (n=5).

Table 1. Key Biochar and Feedstock characteristics

	Biochar	Residue
Fixed Carbon (%)	36.3	14.1
Volatiles (%)	32.9	71.4
Nitrogen (%)	2.7	2.3
Hydrogen (%)	3.4	NA
C:N ratio	22.0	31.9
Sulphur (%)	0.4	NA
$H:C_{org}$	0.59	NA
Ash (%)	28.0	9.6
pH	9.2	5.4
^{13}C enrichment	1.38 ±0.0002 at-%	1.40 ±0.003 at-%
^{15}N enrichment	58.2 ±0.01 at-%	58.5 ±0.03 at-%

(All values provided on an oven dried (60°C) basis. pH was quantified in demineralised water 1:5 w/v)

Table 2: Key soil characteristics (based on soil dry weight)

Soil Texture	
Sand (%)	93.9
Silt (%)	4.3
Clay (%)	1.8
pH ($CaCl_2$)	5.2
SOM (%)	4.6
N-content (mg kg^{-1})	
$N\text{-}NH_4$	1.51 ±0.19
$N\text{-}NO_3$	0.97 ±0.07
P-content (mg kg^{-1})	
$P\text{-}PO_4$	3.96 ±0.17
K-content (mg kg^{-1})	16.49 ±0.92